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STUDIES ON BASOPHILIA OF NUCLEIC ACIDS: THE METHYL GREEN STAINABILITY OF NUCLEIC ACIDS [1]

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A number of reports in the recent cytochemical literature have dealt with the use of the basic dye methyl green as a specific and quantitative stain of desoxyribonucleic acid (DNA). Pollister and Leuchtenberger (1949) first demonstrated by photometric methods the high degree of reproducibility of nuclear methyl green staining in mouse liver and discussed the impairment of stainability caused by pretreatment of slides with hot water. Kurnick (1950a) concluded from test tube experiments that methyl green combines specifically with highly polymerized DNA only. He used the stain for quantitative determinations of DNA in fixed histological material (1950b; Kurnick and Herskowitz, 1951) and to measure depolymerase activity of serum (1950c). Vercauteren (1950) and Devreux *et al.* (1951) also tested the dye-binding capacity of DNA solutions under various conditions and concur with Kurnick's opinion. The methyl green stainability of some types of degenerating chromatin was investigated by Leuchtenberger (1950), Klemperer *et al.* (1950) and Korson (1951), and the influence of x-radiation on stainability of nuclei was tested by Moses *et al.* (1951) and Harrington and Koza (1951).

Impairment of methyl green stainability as it was found to occur in some instances, and as it can be induced by treatment of slides with hot water or mild acid hydrolysis, would, according to Kurnick's view, be due to a depolymerization of the DNA. Pollister and Leuchtenberger (1949) have also used the term "depolymerization" to designate an unspecified change in the configuration of the DNA molecule without implying that depolymerization in the chemical sense (breakage of internucleotide linkages) was necessarily involved.

Although methyl green has long been regarded as an exclusive nuclear stain, there are some indications that it does not possess absolute specificity for DNA, since its ability to distinguish DNA from ribonucleic acid (RNA) depends on the type of fixation used: following fixation in acetic alcohol, staining is entirely restricted to nuclei in many types of tissues, while after fixation in formalin, specificity is often lost and cytoplasmic RNA also binds methyl green strongly. Furthermore, in some tissues RNA is methyl green stainable even after acetic alcohol fixation.

[1] The work reported here started when the author was a Public Health Research Fellow of the National Institutes of Health. At present the author receives support from the University of California Board of Research and from California Cancer Research Funds.

This is notably the case in Nissl substance (as observed by Koenig, and by Swift and Flax, personal communications); we have also found it to occur in the sperm cytoplasm of *Ascaris megalocephala* and in the serous cells of the submaxillary gland of the mouse. In accordance with the concept proposed by Kurnick, it seemed possible that certain types of RNA occur in a highly polymerized state and are therefore able to combine with methyl green. We recorded a hypothesis to that effect in a recent abstract (Alfert, 1951) when we observed that smears of RNA from tobacco mosaic virus, which was known to be highly polymerized, could be stained with methyl green. However, further experiments demonstrated that this hypothesis was inadequate and that other factors, which have not been previously described, strongly influence the methyl green stainability of nucleic acids.

Materials and Methods

The staining reaction of nucleic acids in several animal and one plant tissue was investigated. All tissues were fixed in Carnoy's acetic alcohol and paraffin sections of different materials were mounted together to be treated and stained under identical conditions. Several nucleic acid and nucleoprotein model systems [2] were also used; artificial fibers of nucleohistone prepared from calf thymus were fixed, embedded and sectioned like tissues, and drops of solutions were allowed to dry on slides, fixed in acetic alcohol or ethyl alcohol, and stained. The usefulness of the latter staining models is rather limited because variable amounts of the substrate are likely to be lost from the slides during the staining procedure. Only positive results are significant and constitute at best a qualitative test since a reliable comparison of relative staining intensities is not possible.

Methyl green, Natl. Aniline Div., C.I. No. 685, NG26 was purified by chloroform extraction and dissolved in the phenol-glycerin medium used by Pollister and Leuchtenberger (1949) in a concentration of 0.25%. The pH of this solution is 4.5. Materials were stained for 90 minutes at 37° C., rinsed in distilled water, blotted and differentiated for at least 18 hours in tertiary butyl alcohol. This method results in an excellent and, as shown by photometric measurements, very reproducible stain. For histological purposes we found this staining method superior to that proposed by Kurnick (1950b) since staining is more intense and even throughout the section. Both methods are of equal specificity when tested on the same objects.

The methyl green stainability of all substrates was compared to their general basophilia tested with the basic stain azure B. As described by Flax and Himes (1951), this stain permits visualization of DNA and RNA in different colors, blue and purple, respectively. Occasionally other basic stains were used in aqueous and buffered solutions, and the Feulgen reaction was applied (12 minutes hydrolysis in 1 N HCl at 60° C.).

Removability of the stainable substrates by hot trichloroacetic acid and by specific nucleases was used to insure the specificity of the staining reaction for nucleic acids.

A number of treatments which affect basophilia of nucleic acids were applied to fixed materials prior to staining:

[2] We are greatly indebted to Dr. C. A. Knight as well as to Dr. D. Mazia and members of their laboratories for supplying the nucleic acid and nucleoprotein extracts used in this investigation.

1. Immersion of slides into hot distilled water (90°–95° C.) for 30–60 minutes.
2. Hydrolysis in 1 N HCl at 60° C. for 12 minutes, and immersion in dilute acid (0.01 N HCl) and alkali (0.01 N NaOH) for 24 hours at room temperature.
3. Van Slyke reaction as applied by Ornstein and Flax (unpublished) to remove amino groups of proteins: 10 g. $NaNO_2$ in 45 cc. 50% ethyl alcohol and 15 cc. glacial acetic acid; slides treated for two hours at room temperature.
4. Acetylation of amino groups by acetic anhydride: slides blotted dry out of absolute alcohol and immersed for one hour at room temperature.

A comparison of the relative staining intensity of nuclei was made by means of photometric techniques described elsewhere (Pollister and Ris, 1947; Swift, 1950). The apparatus is similar to that described by Pollister and Moses (1949) and a Beckman B spectrophotometer was used as a light source. The Feulgen reagent was measured at 550 mμ, and methyl green at 630 mμ, slightly off the absorption peak of the dye combined with nucleic acids. Measured values are reported in arbitrary units of dye per nucleus. The tables give mean values and standard errors obtained in several series of measurements.

Experiments and Observations

1. Cytoplasmic basophilia and staining models

Plate 1 contains photomicrographs of four different tissues of a mouse, each stained with azure B and methyl green for comparison. The tissues in the first vertical column—liver, pancreas, submaxillary gland and spinal cord—were stained with azure and the presence of high concentrations of RNA can thereby be demonstrated in all of them. The staining picture of the same tissues with methyl green (second column) is quite different, however; in liver and pancreas the methyl green picture is completely equivalent to a Feulgen preparation of the same material, only nuclei stain and the cytoplasm remains unstained. In the submaxillary gland, however, the cytoplasmic RNA of serous cells combines with methyl green and the same is true for Nissl substance in the motor neurons of the spinal cord. Comparing the two photographs of motor neurons, it is evident that the strongly azurophilic nucleolus does not stain with methyl green. Figure 9 on Plate II is a methyl green preparation of a fertilized egg of Ascaris. In addition to the egg chromosomes and the small sperm nucleus, the whole body of the amoeboid sperm stains strongly with methyl green.

Thus there exist clear-cut differences among ribonucleic acids in different tissues with respect to their ability to bind methyl green. Using model systems of different nucleic acid and nucleoprotein solutions, we were unable to find qualitative differences in stainability. The following systems were investigated:

1. Calf thymus nucleohistone.
2. Calf thymus nucleohistone after irradiation with a dose of 100,000 r (Himes and Alfert, unpublished).
3. Nucleoprotein from sea urchin sperm.
4. 0.1% solution of DNA from chicken erythrocytes.
5. 0.1% solution of yeast RNA (Schwarz).
6. 0.1% solution of tobacco mosaic virus RNA.

7–10. Solutions 3–6 after heating for one hour in a bath of boiling water.

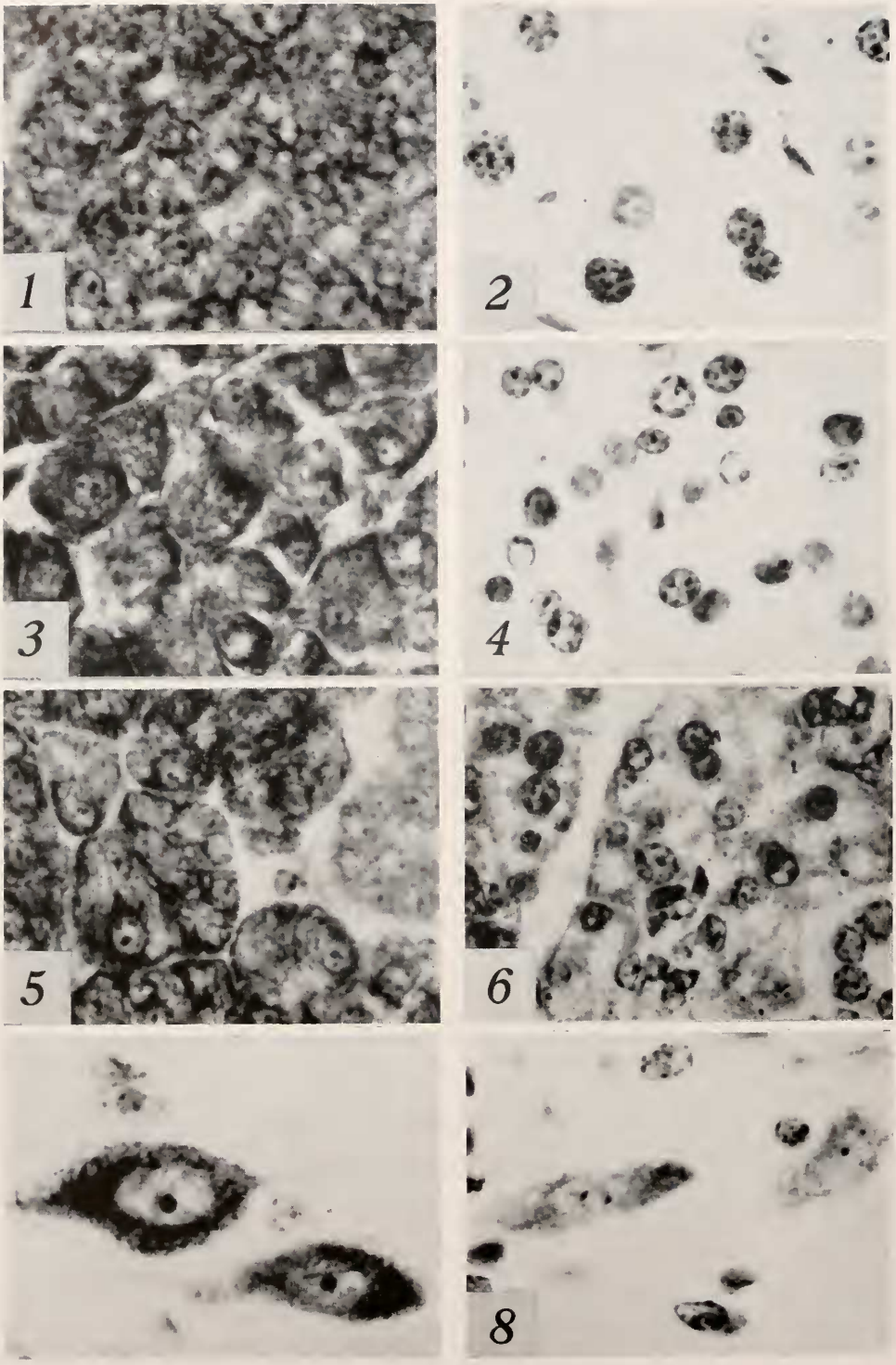

PLATE I. Mouse tissues fixed in Carnoy's acetic-alcohol; 10 μ sections, $\times$ 750.
(Photomicrography by Mr. Victor Duran.)

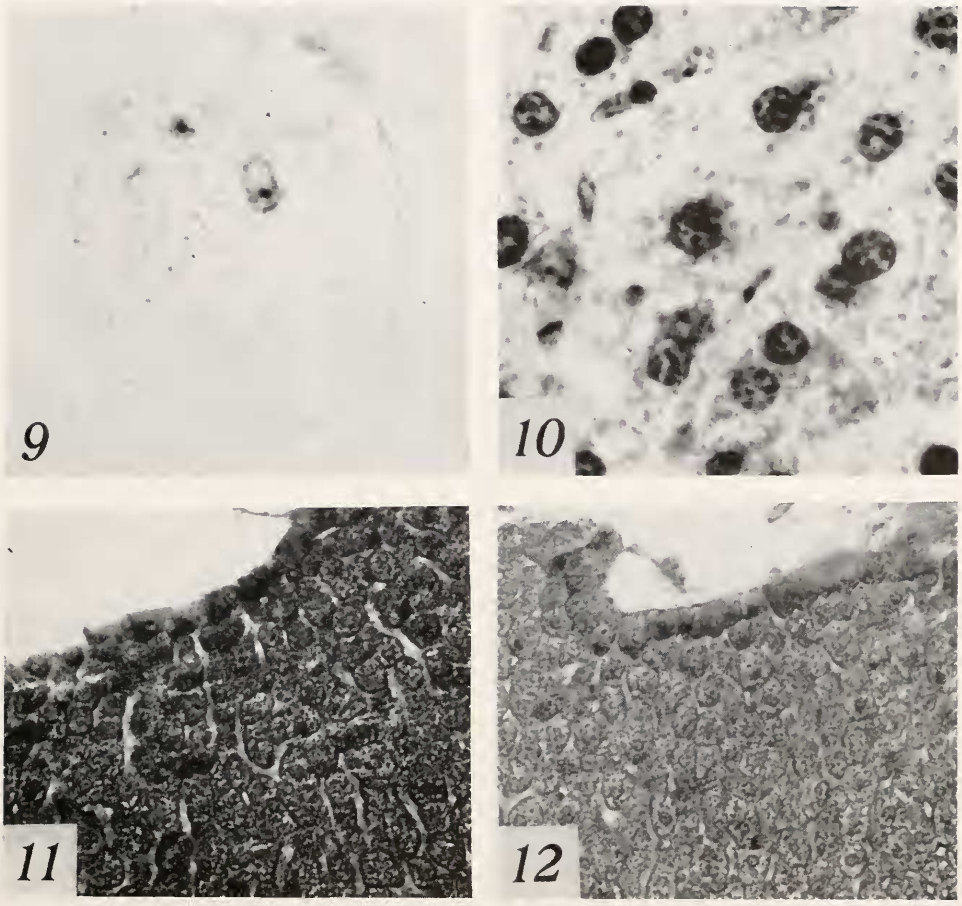

PLATE II. (Photomicrography by Mr. Victor Duran.)

FIGURE 9. Fertilized egg of *Ascaris megalocephala*, Carnoy's, 15 μ section, $\times$ 750; stained with methyl green; Wratten F filter.

FIGURE 10. Mouse liver, as in Figure 2, but first acetylated and then stained with methyl green.

FIGURES 11 and 12. Mouse liver, $\times$ 220; stained with fast green at pH 2; Control (Fig. 11), and after acetylation (Fig. 12).

All these materials stained with methyl green. Fixed smears of isolated cell particulates exhibit staining differences which correspond to those found in histological sections: liver nuclei stain with methyl green and azure, while mitochondria and microsomes stain only with azure but not with methyl green.

If lack or presence of methyl green stainability is not a property of the nucleic acid itself, as these model experiments seem to suggest, a possible clue for the

FIGURES 1 and 2: liver; FIGURES 3 and 4: pancreas; FIGURES 5 and 6: submaxillary gland; FIGURES 7 and 8: motor neurons. Odd numbered figures: stained with azure B; Wratten G filter. Even numbered figures: stained with methyl green; Wratten F filter.

explanation of the observed staining differences in tissues is perhaps provided by the effect of formalin. As mentioned above, fixation in strong formalin (or treatment of Carnoy-fixed material with 50% formalin for three hours) causes loss of methyl green specificity for nuclei. Formaldehyde is known to combine with amino groups of proteins. This reaction could result in making available, for combination with the dye, nucleic acid phosphate groups previously masked by the protein. This hypothesis was tested by subjecting sections to two chemical procedures known to affect amino groups of proteins, the Van Slyke reaction and acetylation by acetic anhydride (see Olcott and Fraenkel-Conrat, 1947). Under the conditions used, these two treatments produce similar results. Their immediate effect consists in a decrease of acidophilia which can be demonstrated by comparing sections stained with fast green at pH 2 before and after treatment (Figs. 11 and 12). The same effect was described by Monné and Slautterback (1951) who used similar procedures. While these reactions result in a loss of some basic groups available for combination with acid dyes, they may at the same time affect the methyl green stainability of nucleic acids; Figure 10 demonstrates that RNA in liver cytoplasm becomes methyl green stainable and that nuclei stain more intensely after acetylation. The treatments also cause the nucleolus in neurons to become methyl green stainable and enhance the stainability of Nissl substance and submaxillary gland cytoplasm and nuclei. Smears of isolated liver mitochondria and microsomes also become methyl green stainable after either treatment. The stainability of ribonucleic acid with azure in any of the mentioned materials is not increased further by these treatments and they do not affect the Feulgen stainability of nuclei.

For unknown reasons the strongly basophilic cytoplasm of the exocrine cells in pancreas does not become methyl green stainable after acetylation or Van Slyke reaction. This probably reflects a fundamental difference in the structure of the nucleoproteins as compared to those of liver cells. Differences in the nucleoproteins of various tissues with respect to their solubility have been described by Koenig and Stahlecker (1952).

Digestion of sections with trypsin in aqueous solution as described by Kaufmann *et al.* (1950) does not produce cytoplasmic methyl green stainability in liver and pancreas. Since the azure stainability was decreased after trypsin digestion, it appears that the enzyme removed the whole nucleoprotein complex in these materials.

The qualitative observations on cytoplasmic staining reported so far indicate that RNA in different tissues may be methyl green stainable or not, and suggest that this is a matter of absence or presence of protein interference. The chemical treatments used bring out further differences in that they may cause an unmasking of stainable groups in some materials but not in others.

2. *Nuclear basophilia*

As far as nuclear staining is concerned, our own data confirm those of Pollister and Leuchtenberger (1949) relating to the reproducibility of the methyl green procedure. Samples of 20 photometric measurements of the amounts of methyl green bound by diploid nuclei of the mouse pancreas were measured on different slides, prepared and stained at different times. The mean values of methyl green content per nucleus thus obtained were quite similar: 100 ± 4, 104 ± 4, and 111 ± 4 (see Table III).

A comparison of the methyl green and Feulgen stainability of tetraploid and diploid nuclei in the liver and pancreas of the mouse was also made and the data are presented in Table I.

Recent work by Ris and Mirsky (1949), Swift (1950) and Leuchtenberger, Vendrely and Vendrely (1951) has demonstrated that photometric Feulgen dye determinations, if done correctly, provide a reliable measure of the DNA content and degree of ploidy of a nucleus. The present data indicate that this is also true for methyl green staining in these two tissues of the same animal, since very similar Feulgen/methyl green ratios are obtained. The average Feulgen/methyl green ratio of these four types of nuclei is therefore a characteristic constant of 2.56 ± 0.07 for these tissues. Measurements conducted under the same conditions on 25 onion root tip nuclei produced a very different Feulgen/methyl green ratio of 1.45 ± 0.03. This indicates that the dye-binding characteristics of this material are not the same as in the previous case.

TABLE I

Amounts of DNA in arbitrary units of Feulgen dye and methyl green in different nuclei*

	Type of nucleus	Number measured	Average amount Feulgen	Average amount methyl green	Ratio Feulgen/methyl green
Mouse pancreas	2n	25	277± 6	100±4	2.77
	4n	15	533±16	212±8	2.52
Mouse liver	2n	15	275± 6	112±4	2.46
	4n	25	597±11	239±6	2.50
					average 2.56±0.07
Onion root tip		25	—	—	average 1.45±0.03**

* To facilitate comparison, the methyl green value for diploid pancreas nuclei obtained in one experiment is set equal to 100; all other values are proportional to that.

** Calculated on basis of optical densities measured consecutively on the same nuclei stained first with methyl green and then by the Feulgen reaction. (The Feulgen/methyl green ratio of mouse tissue nuclei calculated on basis of average optical densities is 2.47±0.05, very similar to that given above which is calculated from average amounts of dye per nucleus.)

It remains to be determined whether a change in the Feulgen/methyl green ratio of a nucleus can be correlated with any particular process—such as depolymerization of the nucleic acid.

a) Treatment with acid and alkali

Taft (1951) criticized the depolymerization concept on basis of the following experiment: soaking of sections in dilute acid and alkali (which he assumed would cause depolymerization of the DNA) resulted in a decreased Feulgen stainability without affecting methyl green stainability of nuclei. The only conclusion that can be drawn from such an observation is that methyl green, as it was used in this case, did not stain DNA specifically at all. We have repeated this experiment and obtained different results: treatment with dilute HCl (pH 2.1) for 24 hours at room temperature did not affect the Feulgen reaction but increased methyl green stainability noticeably. The latter may be due to extraction of histone by HCl (Kurnick,

1950b). Treatment with dilute NaOH (pH 8.1, 24 hours, room temperature) had a profound effect: nuclei were swollen, vacuolated and in various stages of disintegration. Strands of Feulgen positive and methyl green stainable chromatin were scattered throughout the cytoplasm. Although chromatin had been partially dissolved and still retained methyl green stainability, this observation does not constitute a test of the depolymerization hypothesis, since alkali at low temperature can decrease viscosity without depolymerizing DNA (see discussion by Davidson, 1950).

b) Enzymatic digestion

The action of desoxyribonuclease results in depolymerization and loss of DNA from sections. The effectiveness of enzymatic digestion depends on the type of fixation and also differs in various materials, depending probably greatly on the compactness of the nuclei. Under the conditions which we used, interphase nuclei in many mouse and Ambystoma tissues were Feulgen and methyl green negative after digestion for 40 minutes at room temperature. Some nuclei in sections of Ambystoma spleen, however, retained Feulgen and methyl green stainability after being exposed to enzyme for as long as 90 minutes at room temperature. Used at low temperature and for a shorter time, the enzyme decreased methyl green stain-

Table II

Effect of desoxyribonuclease (0.15 mg./cc. at pH 7 for 30 minutes at 4° C.) on stainability of tetraploid mouse liver nuclei

	Number measured	Average amount Feulgen	Average amount methyl green	Ratio Feulgen/ methyl green
Control	25	597±11	239±6	2.50
After DNA-ase	15	457±15	142±11	3.72
Reduction by enzyme		23%	41%	

ability more rapidly than Feulgen stainability in mouse liver nuclei (Table II). This effect was also noted by Leuchtenberger, Himes and Pollister (1949).

c) Effect of hot water and hot acid hydrolysis

Treatment of sections with hot water and Feulgen hydrolysis (1 N HCl at 60° C. for 12 minutes) removes methyl green stainability of DNA. However these treatments also affect the azure stainability of DNA in a similar way; the effect produced is therefore likely to have a more general basis than the peculiar specificity of methyl green. We observed that basophilia of nucleic acid can be partially restored in these cases by subjecting sections to acetylation, the Van Slyke reaction or trypsin digestion. Photometric measurements on several experimental series are presented in Table III.

All determinations were made on diploid pancreas cells in which the treatments used do not produce cytoplasmic staining which would interfere with nuclear measurements. Acetylation or Van Slyke reaction initially increase nuclear stainability approximately 50%. Exposure to hot water produces a small but significant drop of 15% of the Feulgen values while methyl green stainability is

reduced to the level of the surrounding cytoplasm (about 95% transmission of the incident light). Following hot water treatment either of the protein reactions will restore stainability to an extent at least as great or greater than the initial increase it produces in unheated slides; and if the protein reactions precede hot water treatment they protect from loss of stainability to the same extent. At any stage of these treatments nuclear methyl green stainability is removable by desoxyribo-

TABLE III

Effects of various treatments on methyl green and Feulgen stainability of 2n mouse pancreas nuclei; each value is a mean of 20 measurements

	Methyl green			Feulgen
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 1
Control	100±4	104±4	111±4	277±6
After acetylation	156±8	—	161±3	267±6
After Van Slyke	—	142±4	140±4	—
After hot water	0	0	0	234±4
After hot water and acetylation	86±4	—	68±4	—
After hot water and Van Slyke	—	85±4	71±3	—
After acetylation and hot water	—	—	69±3	—

nuclease but resistant to ribonuclease. The azure stainability of nuclei appears to follow the same pattern in the course of these treatments but loss of stainability is never complete. Photometric measurements were not made. Sections of nucleohistone fibers also show similar effects but are more resistant to loss of basophilia than interphase nuclei; they show a less pronounced decrease after hot water treatment and remain methyl green stainable even after Feulgen hydrolysis.

Discussion and Conclusions

It is necessary to consider the validity of various model systems and test tube experiments which have been used to investigate the mechanism of methyl green staining. Obviously any model system represents a simplification of the very complex conditions that obtain in tissues and the observations made on such models must be interpreted with great caution. The conditions under which the model system is tested will also influence the results. This is demonstrated by the data reported by Kurnick (1950a); in one experiment the dye-nucleic acid complex was precipitated with alcohol and ribonucleic acid was found to combine with an appreciable amount of methyl green. When lanthanum was used to precipitate the dye-NA complex, only highly polymerized DNA retained methyl green. Since staining of tissues is not ordinarily performed in presence of lanthanum, the significance of this observation with respect to the histological use of the stain cannot be evaluated.

In our own experiments the various model systems were stained in the same way as sections of fixed tissues. Under these conditions the dye was found to be unable to distinguish RNA from DNA. Although this constitutes no definite proof, it suggests that the specificity of methyl green for DNA which undoubtedly exists

in many tissues is not primarily due to an innate inability of the dye to combine with RNA. Staining experiments on tissues furthermore demonstrate that stainable groups in nucleic acids are made available for dye combination by treatments which are known to affect proteins in definite ways. At this point it is not yet possible to suggest a definite mechanism for the protein interference, since removal of amino groups could affect the nucleoprotein in a number of ways. We know from the work of Kaufmann *et al.* (1951), Mirsky and Ris (1951) and Pollister *et al.* (1951) that masking of stainable groups is a general phenomenon which may affect the staining picture obtained with various dyes. Methyl green seems to differ from other basic dyes mainly in being more sensitive to protein interference.

To account for the behavior of methyl green, Kurnick (1950a) assumed the operation of stereochemical factors. On the basis of *in vitro* experiments he claims that all triphenylmethane dyes with two methyl amino groups, such as methyl green, ethyl green and malachite green, exhibit the same type of selectivity. However one sample of Grübler's malachite green which we tested as a tissue stain did not behave like methyl green : it stained liver cytoplasm as well as nuclei and produced a staining picture similar to the nonspecific methyl violet. Pollister and Leuchtenberger (1949), on the other hand, have pointed out that methyl green carries two positive charges and may therefore require phosphate groups at definite spacings for stable combination. Adopting this view for a possible explanation of methyl green selectivity, we may assume that ribonucleoprotein complexes in many tissues are of a type in which the necessary phosphate groups are masked by protein; but in other cases, such as Nissl substance, methyl green is able to combine either because of the different nature of the complex or because of the presence of some relatively "free" RNA.

DNA in nuclei normally combines to a definite extent with methyl green, resulting in a characteristic Feulgen/methyl green ratio. Changes in this ratio can be brought about in a number of ways and impairment of methyl green stainability is not necessarily related to depolymerization. Even the effect of desoxyribonuclease cannot be interpreted in a clear-cut way. The enzyme may simply remove certain exposed groups necessary for methyl green combination at a faster rate than the many more numerous nucleotides which contribute to the Feulgen reaction. Treatment with hot water and Feulgen hydrolysis affect basophilia of DNA in general and the observations suggest that masking of stainable groups is at least partially responsible. A similar conclusion was reached by Herrmann *et al.* (1950) who found that the toluidine blue binding capacity of RNA is impaired when the nucleic acid is heated in the presence of protein. They attribute this effect, which was observed *in vitro,* to the masking of staining sites by basic groups of protein which had become exposed in the course of heating. In our experiments we have never succeeded, however, in restoring maximum stainability in heat-treated sections. This may be due to a low efficiency of the protein reactions under the conditions used; since there is some loss of Feulgen stainability in the course of heating it is also possible that a small loss of the Feulgen reactive material is responsible for a much greater loss of methyl green stainability. The mammalian nuclei which we used have a notoriously low RNA content and staining of nuclear RNA can hardly play an important role in this case. This is borne out by our observations on the effect of digestion by nucleases. However in other types of material staining of nuclear RNA may be an important variable.

For histological purposes methyl green is often used in combination with pyronin, the well known Unna-Pappenheim mixture. This can be used as a satisfactory qualitative method to distinguish RNA and DNA in many tissues. As a precise and quantitative histochemical technique for DNA determinations, methyl green is much less satisfactory than the Feulgen reaction which appears to be less subject to modifying influences. In combination with the Feulgen reaction, methyl green is a useful tool which serves to characterize a particular nucleoprotein complex in terms of a Feulgen/methyl green ratio. Changes of this ratio are of interest but they cannot be interpreted in a precise way.

Summary

1. The capacity of nucleic acids to combine with basic dyes under various conditions was investigated by means of fixed tissues and model systems. Observations and experiments suggest that the specificity for nuclear DNA which methyl green exhibits in many, but not all, tissues is caused by blockage of stainable groups of ribonucleic acid by protein.

2. Nuclear staining with methyl green is also subject to modification by changes in the degree of protein interference, and impairment of stainability cannot be correlated unequivocally with depolymerization of DNA.

3. Since variable degrees of RNA staining, differences in the extent to which DNA staining is affected by protein interference, and possibly changes in the degree of polymerization of the DNA may all influence the staining intensity of nuclei, methyl green staining is not a very suitable method for quantitative determination of DNA in cell nuclei.

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